AD______________

Award Number: W81XWH-10-1-0422

TITLE: Targeting Cell Surface Proteins in Molecular Photoacoustic Imaging to Detect Ovarian Cancer Early

PRINCIPAL INVESTIGATOR: Charles Drescher, M.D.

CONTRACTING ORGANIZATION: Fred Hutchinson Cancer Research Center
Seattle, WA 96109

REPORT DATE: July 2013

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE
July 2013

2. REPORT TYPE
Final

3. DATES COVERED
1 July 2010 - 30 June 2013

4. TITLE AND SUBTITLE
Targeting Cell Surface Proteins in Molecular Photoacoustic Imaging to Detect Ovarian Cancer Early

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0422

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Charles W. Drescher

E-Mail: cdresche@fhcrc.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Fred Hutchinson Cancer Research Center
1100 Fairview Ave N, J6-300
Seattle, WA 98109-4433

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Our idea is to apply a series of novel techniques to identify the reagents needed to move imaging technology forward into the clinic. While molecular imaging strategies are now approaching the resolution required to detect ovarian cancer in an early curable stage, specific imaging probes are not currently available and are urgently needed to realize the potential of imaging for ovarian cancer early detection. To address this challenge we are undertaking a comprehensive proteomic analysis of the cell surface membrane of ovarian cancer cells. We have completed a survey of the cell surface N-linked glycoproteome of OVCAR3 cells and serous ovarian cancer cells isolated from ascites using a novel biochemical labeling method that allows for highly selective capture and internal validation of candidate peptides and proteins by LC-MS/MS. A total of 519 N-glycoproteins were identified, of which 411 were associated with the cell surface based on bioinformatics methods. Included in this list are a number of established ovarian cancer cell surface proteins such as MUC16 and mesothelin. These data provide a catalogue of the cell surface N-glycoproteome of ovarian cancer cells. We further annotated our list to prioritize surface proteins as candidate targets for molecular imaging probes and use immunohistochemistry to confirm FOLR1 as being strongly and specifically expressed on the surface of tumor cells in ovarian cancer tissues and provide new evidence that FOLR1 expression is absent in normal ovary and fallopian tube tissue. Consequently FOLR1 represents a promising imaging target for detecting localized ovarian cancer.

15. SUBJECT TERMS
Ovarian Cancer, Molecular Imaging, cell surface proteome, N-glycoproteins, FOLR1

16. SECURITY CLASSIFICATION OF: a. REPORT	b. ABSTRACT	c. THIS PAGE	17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
U	U	U	UU	17	USAMRMC

19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION:
To achieve the promise of ovarian cancer early detection molecular imaging tests are urgently needed. Currently available tests such as transvaginal sonography, CT and MRI lack requisite sensitivity and specificity to detect ovarian cancer in an early curable phase. Integrating advances in molecular biology, nanotechnology, and imaging technology, molecular imaging utilizes specific probes as contrast agents to visualize cellular processes at the molecular level. Molecular imaging represents a paradigm shift away from traditional strategies that rely on detecting nonspecific physical properties and anatomic change. The approach holds great promise for early detection because molecular alterations frequently precede anatomic changes during cancer development. One of the greatest obstacles to applying molecular imaging strategy to the problem of ovarian caner detection is the lack of suitable markers to target. This proposal addresses this critical bottleneck by using a novel proteomic strategy to identify and validate proteins that are abundantly and specifically expressed on the luminal surface of ovarian cancer cells as these proteins are ideal molecular imaging targets.

KEYWORDS:

- Ovarian Cancer
- Molecular Imaging
- Cell surface proteome
- N-glycoproteins
- FOLR1

OVERALL PROJECT SUMMARY:

<u>Aim 1)</u> Identify ovarian cancer associated surface proteins from the set of proteins detected in a comparative LC-MS/MS-based analysis of the surface proteome of serous ovarian cancer and human ovarian surface epithelial cells.

<u>N-glycoprotein cell surface capture (CSC) method:</u>
A modification of the method of Wollscheid B. et. al (*1*) was used to label, digest and capture N-glycoproteins from the surface of OVCAR3 cells and serous ovarian cancer cells isolated from ascites. Briefly $7x10^7$ – $3.5x10^8$ ovarian cancer cells were washed twice with labeling buffer (0.1% FBS in PBS). Cis-diol groups of carbohydrate moieties associated with surface glycoproteins were oxidized to aldehydes by incubation with 2.5 mM sodium meta-periodate (Sigma) for 30 minutes at room temperature with gentle rocking. Oxidized cells were washed twice with labeling buffer and subsequently labeled with the bi-functional linker biocytin hydrazide (Biotium, Inc.) at a concentration of 6.5 mM for 30 minutes at room temperature with gentle rocking. Labeled cells were incubated with 25 mM tyrosine hydrazide (Sigma) for 10 minutes to prevent the formation of internal Schiff bases with primary amines and then washed twice with labeling buffer. Cells were lysed in hypotonic lysis buffer with complete mini protease inhibitors (Roche) and 5 mM cyanoborohydride (Sigma) and homogenized using a dounce homogenizer. Membrane proteins were isolated by sequential ultracentrifugation and sonicated using a microtip sonicator and subsequently reduced and alkylated with 2 mM Tris (2-carboxyethyl) phosphine hydrochloride, TCEP (Sigma) and 10 mM iodoacetamide for 30 minutes each at 4°C. Membrane proteins were then sonicated and digested with TPCK Trypsin (Thermo Scientific) for at least 1 hour or overnight at 37°C on a shaker. A silver stained protein gel was used to confirm the extent of digestion and if incomplete, additional trypsin was added and the sample was incubated overnight again at 37°C on a shaker.

Following digestion, peptides were incubated with strepdavidin beads (Thermo Scientific) for 2-3 hours at room temperature and then over night at 4°C. A dot blot was used to confirm that all labeled peptides had bound to the beads. If unbound, labeled peptides were still present, additional beads were added and incubation was repeated. Beads and adherent peptides were washed and incubated for 30 minutes at 65°C in 2% SDS (*2*). After

a series of additional washes peptides were lyzed from the beads by incubation with PNGaseF (New England Biolabs) over night at 37°C on a shaker. Released glycopeptides were collected and dried down with a Speedvac concentrator on low setting for ~4 hours followed by two washes in 50% methanol. Dried peptides were later dissolved in 0.1% formic acid for analysis by LC-MS/MS. Duplicate experiments were performed using 2 independent aliquots of both OVCAR3 and SOC cells.

Peptide and protein identification by LC-MS/MS:

Protein identification was performed using methods described previously (*3*, *4*). Briefly, each sample was individually analyzed by LC-MS/MS using a LTQ-ORBITRAP mass spectrometer (Thermo-Finnigan) coupled to a nanoflow chromatography system (Eksigent) over a 90 minute linear gradient. Acquired tandem mass spectra were searched using X!Tandem (*5*) against a combined International Protein Index database (IPI) of the forward and reverse sequence released on August, 2010 (IPI.human.v.3.75). Peptide spectra match (PSM) confidence was determined by calculating the false discovery rate (FDR) where PSMs are ranked by the Tandem expect score. Peptides with PSM corresponding to a FDR <0.01 were selected for further analysis. All selected peptides were evaluated for the presence of an N-linked glycoprotein motif (N~XS/T) and whether they demonstrated an expected mass shift associated with PNGase-F digestion. Peptides were grouped into protein groups using ProteinProphet (*6*) and protein groups with ProteinProphet probability >0.9 were selected for further analysis. Protein groups include either unique proteins or multiple proteins (IPI sequences) with shared peptides that are supported by the peptide evidence. Each protein group is assigned a pseudo-abundance score based on their spectral count (*7*), calculated as the number of PSMs assigned to members of each protein group. Protein groups identified in each sample were then aligned using previously described methods (*8*) and gene-symbol(s) were assigned. The result is an analytic data set describing the protein groups and pseudo-abundance score for each sample (*8*). The code for generation of the alignments is available at http://proteomics.fhcrc.org.

Annotation of cell surface proteins:

The following features were used to annotate proteins as being derived from the cell surface: a) the presence of a N~XS/T motif, b) cell membrane localization as defined by the Gene Ontology (GO) Cellular Component database, c) the presence of transmembrane helices as determined by the TMHMM prediction program (http://www.cbs.dtu.dk/services/TMHMM/) and d) experimental evidence supporting a cell surface origin identified by review of Pubmed, Genecards and Information Hyperlinked over Proteins (www.ihop-net.org/UniPub/iHOP/) databases. GO defined cellular components for each protein sequence were determined using the generic GO slim (version 1.2) from the GO consortium (http://www.geneontology.org/GO.slims.shtml). GO slim files are reduced ontologies with significantly fewer categories than the complete GO ontology. The script "map2slim" (available from GO) was used to assign proteins to their nearest GO category and to identify those that are located in the extracellular or plasma membrane. Protein sequences were uploaded to the TMHMM Server v. 2.0 and the prediction algorithm was used to identify proteins expected to contain membrane spanning regions. Proteins possessing all 3 characteristics (contains a N~XS/T motif, associated with plasma membrane based on GO cellular component and contains at least one transmembrane helices by TMHMM prediction) were characterized as cell surface proteins. Proteins that lacked 1 or more of these features were also characterized as cell surface proteins if there was strong experimental evidence for a cell surface origin based on literature review.

Cell surface proteins were further annotated into their molecular functional groups as defined by PANTHER (**P**rotein **AN**alysis **TH**rough **E**volutionary **R**elationships). Molecular function terms defined by PANTHER are based on the evolutionary relationships of genes and also correspond to terms associated with Gene Ontology (*9*). These terms can be downloaded from the PANTHER website (http://www.pantherdb.org/) in the same format as the generic GO slim terms from the GO consortium, and the script "map2slim" was then used to assign the molecular functions to each protein.

Results for Aim 1:
Our goal was to discover ovarian cancer molecular imaging targets by selective capture of plasma membrane glycoproteins from ovarian cancer cells using 1) a cell culture model of ovarian cancer and 2) SOC cells collected from untreated patients at the time of surgery. Plasma membrane glycoproteins were selected through biotinylation with biocytin hydrazide. This reagent was covalently coupled to the oligosaccharides attached to polypeptide side-chains of extracellular membrane proteins on living cells after oxidization of extracellular sugar residues. After protein extraction and trypsin digestion, derivatized glycopeptides were captured on streptavidin beads. Proteins were then enzymatically released with PNGase-F and prepared for MS analysis. This method provides additional specificity by allowing removal of culture media containing plasma and secreted proteins in addition to selecting extracellular proteins away from other cellular glycoproteins.

Analysis of the CSC fractions from two biologic replicates of cultured OVCAR3 and SOC isolated from ascites identified a total of 772 unique proteins among the 471 and 475 total proteins identified from analysis of OVCAR 3 and SOC cells respectively (**Table 1**). Overall, 67% (519/772) of these proteins contained at least one peptide with an N~XS/T binding motif and demonstrated a mass shift associated with PNGaseF digestion and thus are confirmed N-glycoproteins. Overall 411 of 772 (53.2%) all proteins identified were characterized as being derived from the cell surface based on our classification methods. This includes 394 of 519 (75.9%) proteins containing an N~XS/T motif. Only 17 of 253 (6.7%) proteins without an N~XS/T motif were classified as being associated with the cell surface. The high proportion of cell surface proteins among N~XS/T motif containing proteins validates the selectivity of the CSC method.

The overlap between cell surface proteins identified from analysis of OVCAR3 and SOC cells was modest (141 of 411 proteins) (**Table 1**). A majority of the cell surface proteins from both cell types mapped to molecular function categories known to be associated with the plasma membrane. Compared to a list of proteins identified by LC-MS/MS analysis of OVCAR3 whole cell lysates previously reported by our group (*4*) proteins identified in CSC fractions were highly enriched for receptor, transport and ion channel activity molecular function groups and greatly reduced in translation, transcription and enzyme regulator activity (**Figure 1**). A comprehensive list of all surface proteins identified in each cell type annotated with information on protein IDs, spectral count, presence or absence of a N~XS/T motif, presence of transmembrane helix, GO cellular localization, PANTHER molecular function group and normal tissue expression is provided as Supplementary Material (**Table S1, available upon request**).

Aim 2) Prioritize ovarian cancer-associated surface proteins for their utility as molecular photoacoustic imaging targets and validate their expression in ovarian cancer tissues by immunohistochemistry. Generate high affinity scFv to 3-5 of the most promising markers.

Prioritizing cell surface proteins as candidate molecular imaging targets:
The surface protein dataset was integrated with several comprehensive protein and gene expression databases available to our group including, a) gene expression data in serous ovarian cancer relative to normal ovary and fallopian tube (kindly provided by PO Brown; data generated using Stanford HEEBO arrays containing 44,544 70-mer probes and printed at Stanford Functional Genomics Facility. Details on the arrays and the protocols used are available on the Stanford Functional Genomics website (http://www.microarray.org/sfgf/), b) 889 confident plasma proteins identified by the Plasma Proteome Project, (c) normal plasma proteins as derived from a list of approximately 4900 IPIs identified in the plasma of cancer-free women (derived from over 1,000 MS/MS interrogations of highly fractionated plasma) (*10-12*), (d) over 3,500 proteins identified in cancer ascites fluid (a fluid proximal to a tumor) (*13*), (e) transcript signatures derived from a normal human tissue expression data set including 36 types of normal human tissues (*14*) and f) data on normal tissue protein expression available from The Human Protein Atlas website (www.proteinatlas.org). The Human Protein Atlas project provides a comprehensive antibody-based expression and localization profile for over 10,000 human protein coding genes in 66 cell types from 48 normal human tissues and in 20 cancer types (*15*).

Cell surface proteins of high rank had the following criteria 1) highly abundant on the surface of OVCAR3 or SOCs based on spectral count, 2) over-expressed in serous ovarian cancer tissue relative to normal ovary and/or fallopian tube tissue based on transcript arrays 3) lowly abundant or absent in plasma and ascites fluid and 4) low or absent expression in normal somatic tissues based on gene expression arrays and public data available on The Human Protein Atlas website. The normal tissue gene expression profile dataset was modified and processed as described by Fang (*8*) and mean intensities and standard deviation of expression level across all normal tissues was determined for each of 12218 genes. For each gene we identified tissues with "high expression" of that gene defined as an expression level exceeded 1 standard deviation above the median level for all genes in all tissues.

Immunohistochemical (IHC) staining for validating membrane expression of selected surface proteins in ovarian cancer tissues:

IHC staining of tumor tissue collected from patients with serous ovarian cancer (*n*=10) and tissue from patients with benign ovarian tumors and pre and post-menopausal patients with normal ovaries and fallopian tubes (*n*=5 each) was performed for 6 candidate targets (FOLR1, MFGE8, ITGB6, ST-14, CSPG5 and TACSTD2) for which commercial antibodies were available. Staining for FOLR1 (NCL-L-FRALPHA, Leica Microsystems, Wetzlar, Germany), MFG-E8 (LB-B3130, Lifespan Biosciences, Seattle, WA), ITGB6 (21703-1-AP, Proteintech Group, Chicago, IL), ST-14 (LSB4906 Lifespan Biosciences, Seattle, WA) and CSPG5 (AF5685 R&D Systems, Minneapolis, MN) was performed on the Ventana Discovery XT autostainer using standard CC1 antigen retrieval following bench-top deparaffinization (Ventana, Tucson, AZ). The antibodies were diluted in antibody diluent (Ventana) and incubated at room temperature for 1 hour followed by a 32 minute room-temperature incubation with either biotinylated goat-anti-mouse IgG (115-065-003, Jackson Immunoresearch, West Grove, PA) or biotinylated goat anti-rabbit IgG (111-065-144, Jackson). The proteins were then detected using the DABMAP kit (Ventana) and the slides counterstained with hematoxylin (Ventana) and coverslipped with Cytoseal 60 (Richard Allan, Kalamazoo, MI) following washing and dehydration. Staining for TACSTD2 (AF650, R&D Systems, Minneapolis, MN) was performed manually using the Anti-Goat HRP-DAB Cell & Tissue Staining Kit (CTS008, R&D Systems) and Anti-Sheep HRP-DAB Cell & Tissue Staining Kit (CTS019, R&D Systems) respectively following manufacturer's instructions. The antibodies were incubated 1 hour at room temperature. Slides were counterstained with hematoxylin (Biocare) and coverslipped with Cytoseal-60 (Richard Allan) following dehydration.

Results for Aim 2:

The list of cell surface proteins was prioritized in order to identify candidate targets for molecular imaging probes using criteria outlined above. In order to facilitate validation of the candidates we limited this analysis to proteins for which antibodies suitable for IHC were reported to be commercially available. Six candidate targets and associated annotation are presented (**Table 2**). FOLR1, ITGB6, TACSTD2, CSPG5 and ST14 were all found to be at least 2x over-expressed at the mRNA level in serous ovarian cancer relative to site-matched normal tissue (i.e. ovary or fallopian tube) and demonstrate "high expression" in only a limited number of normal tissue types. Protein level expression data for ITGB6 was available from The Human Protein Atlas website and demonstrated expression in 35 of 66 normal tissue types tested. We included MFGE8 as a potential target based on strong experimental data in the literature indicating MFGE8 protein may be a diagnostic marker for ovarian cancer (*16*) and present in only a small subset of normal tissue types (13 of 64 normal tissues tested by Human Protein Atlas investigators).

IHC confirmed cell surface expression in human serous ovarian cancers for 4 of the candidates tested (FOLR1, MFGE8, ITGB6 and TACSTD2) (**Figure 2**). Cell surface protein expression for ST14 and CSPG5 was either weak or highly variable using available antibodies (data not shown). Further studies using tissue from benign ovarian tumors and normal ovarian and fallopian tube demonstrate cancer specific expression for FOLR1 (**Figure 3**). All other markers demonstrated significant expression in either benign tumors or normal ovarian and fallopian tube or both (**Table 3**).

Overall Discussion:
CSC is a highly selective method for labeling the cell surface N-glycoproteome of intact viable cells. The identification by mass spectrometry of peptides containing a consensus N-linked glycoprotein motif (N~XS/T) and demonstrating an expected mass shift induced by PNGase-F digestion provides internal confirmation that the detected peptide is derived from a labeled protein. A limitation of the approach is the large amount of input material that is required which generally restricts analysis to cells that either grow robustly in culture or can be readily isolated from multiple samples and pooled. As a result, we were not able to generate sufficient amounts of normal material such as human ovarian surface cells or fallopian tube epithelial cells to use as comparison group for our analysis. A second limitation is that other glycoproteins such as O-linked glycoproteins are not captured by the approach.

A total of 411 cell surface proteins were identified. As reported in the Supplementary Materials these include a number of well validated ovarian cancer cell surface proteins such as MUC16, DSG2, and mesothelin. Prior work using the Sulfo-NHS-SS-BIOTIN method to label OVCAR3 cells identified a total of 700 protein groups of which roughly 25% were associated with the cell membrane based on Gene Ontology cellular location annotations (*4*). The overlap between this previously reported list of surface proteins and the current list is modest, as can be expected based on the different biochemical capture methods used. Only 47 of the surface protein groups identified by CSC analysis of OVCAR3 were found by the Sulfo-NHS-SS-BIOTIN method, suggesting each approach identifies unique subsets of proteins.

We identified from our candidates FOLR1 as a particularly promising target for molecular imaging of ovarian cancer. FOLR1 protein is a folate receptor family member that binds folic acid and a number of reduced folic acid derivatives and transports 5-methyltetrahydrofolate into cells (*17*). FOLR1 transcript expression is up-regulated in a number of cancers including ovarian, breast and colorectal cancer. Large series involving IHC analysis of hundreds of samples demonstrate FOLR1 protein is expressed in 75-97% of EOC and is associated with serous histology (*18*). Interestingly, where it has been reported, FOLR1 expression by IHC demonstrates a predominately cytoplasmic or cytoplasmic and nuclear staining pattern (*19*). This could be due to many factors including antibody specificity and/or recycling rates of the receptor between the membrane and intra-cellular pool. Our data demonstrate that significant cell surface FOLR1 expression exists in ovarian cancer cells. Importantly the absence of expression of FOLR1 in normal ovary and FT suggests it may be a useful target for detecting localized ovarian cancer. Validation studies that include a larger number and spectrum of normal and benign tissues and early stage and occult ovarian cancers are ongoing.

KEY RESEARCH ACCOMPLISHMENTS:

- Catalogued the N-linked cell surface glycoproteome of OVCAR 3 cells and serous ovarian cancer cells isolated from ascites. A total of 519 unique proteins were identified of which 411 were established to be associated with the cell surface based on bioinformatics methods.

- Prioritized cell surface proteins for utility as molecular imaging probes by integrating cell surface proteome data with comprehensive gene expression and proteomic datasets generated from ovarian cancer and normal tissues and plasma.

- Confirmed ovarian cancer cell surface expression by immunohistochemistry for 4 of 6 lead candidates (FOLR1, MFGE8, ITGB6 and TACSTD2)

- Identified FOLR1 as a high priority target for ovarian cancer molecular imaging probes.

CONCLUSION:

We generated for the first time a comprehensive list of cell surface N-glycoproteome of ovarian cancer cells. Based on success in other cancers (e.g. Herceptin for breast cancer and Rituxin for lymphoma and leukemia) the identified ovarian cancer surface proteins are potential ovarian cancer diagnostic and therapeutic targets. We annotated these proteins to specifically prioritize them as candidate targets for molecular imaging probes. Immunohistochemistry confirms ovarian cancer cell surface expression for 4 of 6 candidates tested, including FOLR1, a molecule that is currently under clinical investigation as a therapeutic target for patients with ovarian cancer. We found FOLR1 expression to be specific to ovarian cancer cells with little or no expression in surrounding tissues including normal ovary and fallopian tubes or benign ovarian tumors suggesting it may be a useful target for ovarian cancer early detection. The identification of FOLR1 as a potential target for ovarian cancer imaging validates our approach and provides motivation for further credentialing of other candidate markers we have identified.

Our overall goal is to develop nanoparticle-based molecular imaging probes that can be used in conjunction with photoacoustic imaging (PAI) as an ovarian cancer early detection test. Dr. Drescher has been collaborating with Dr. Sanjiv Gambhir, Chairman Department of Radiology and Head of Molecular Imaging Program at Stanford University and together these investigators are developing a full project for inclusion in the Pacific Ovarian Cancer Research Consortium Ovarian Cancer (POCRC) SPORE competing renewal application due September 2013. The project entitled “Molecular Photoacoustic Imaging to Detect Ovarian Cancer Early” includes as specific aims the first-ever clinical trial of PAI for ovarian imaging in healthy women and women with ovarian cancer and pre-clinical development of FOLR1 targeted nanoparticle imaging probes including small animal imaging experiments.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Lay Press:

CDMRP Ovarian Cancer Research Program 2013, Early Detection and Diagnosis, highlighted project.

Peer-Reviewed Scientific Journals:

N-glycoprotein cell surface capture proteomics identifies FOLR-1 as a promising target for molecular imaging of ovarian cancer. C. Drescher, A. Green, Q. Fang et. al. under revision- Clinical Cancer Research

Presentations:

Canary Foundation 8th Annual Early Detection Symposium, May 7, 2012, Stanford University, Palo Alto, California

REPORTABLE OUTCOMES:

1. Catalogue of N-linked cell surface glycoproteins on ovarian cancer cells.

2. Annotation of cell surface proteins as candidate targets for molecular imaging probes.

3. Confirmation of FOLR1 as a target for ovarian cancer early detection using molecular imaging.

OTHER ACHIEVEMENTS:

Data generated from this award represents a key step in translating the potential of molecular photoacoustic imaging for ovarian cancer detection to the clinic. These data are being used to support development of one of four full projects in the soon to be submitted competing renewal POCRC SPORE portfolio.

REFERENCES:

1. B. Wollscheid *et al.*, Mass-spectrometric identification and relative quantification of N-linked cell surface glycoproteins. *Nature biotechnology* 27, 378 (Apr, 2009).
2. R. J. Austin *et al.*, Proteomic analysis of the androgen receptor via MS-compatible purification of biotinylated protein on streptavidin resin. *Proteomics* 12, 43 (Jan, 2012).
3. V. Faca *et al.*, Contribution of protein fractionation to depth of analysis of the serum and plasma proteomes. *Journal of proteome research* 6, 3558 (Sep, 2007).
4. V. M. Faca *et al.*, Proteomic analysis of ovarian cancer cells reveals dynamic processes of protein secretion and shedding of extra-cellular domains. *PLoS ONE* 3, e2425 (2008).
5. B. MacLean, J. K. Eng, R. C. Beavis, M. McIntosh, General framework for developing and evaluating database scoring algorithms using the TANDEM search engine. *Bioinformatics* 22, 2830 (Nov 15, 2006).
6. A. I. Nesvizhskii, A. Keller, E. Kolker, R. Aebersold, A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem* 75, 4646 (Sep 1, 2003).
7. A. Gilchrist *et al.*, Quantitative proteomics analysis of the secretory pathway. *Cell* 127, 1265 (Dec 15, 2006).
8. Q. Fang *et al.*, Brain-specific proteins decline in the cerebrospinal fluid of humans with Huntington disease. *Mol Cell Proteomics* 8, 451 (Mar, 2009).
9. H. Mi *et al.*, PANTHER version 7: improved phylogenetic trees, orthologs and collaboration with the Gene Ontology Consortium. *Nucleic Acids Res* 38, D204 (Jan, 2010).
10. S. J. Pitteri *et al.*, Detection of elevated plasma levels of epidermal growth factor receptor before breast cancer diagnosis among hormone therapy users. *Cancer Res* 70, 8598 (Nov 1, 2010).
11. R. L. Prentice *et al.*, Novel proteins associated with risk for coronary heart disease or stroke among postmenopausal women identified by in-depth plasma proteome profiling. *Genome Med* 2, 48 (2010).
12. S. J. Pitteri *et al.*, Postmenopausal estrogen and progestin effects on the serum proteome. *Genome Med* 1, 121 (2009).
13. L. M. Amon *et al.*, Integrative proteomic analysis of serum and peritoneal fluids helps identify proteins that are up-regulated in serum of women with ovarian cancer. *PLoS One* 5, e11137 (2010).
14. X. Ge *et al.*, Interpreting expression profiles of cancers by genome-wide survey of breadth of expression in normal tissues. *Genomics* 86, 127 (Aug, 2005).
15. M. Uhlen *et al.*, Towards a knowledge-based Human Protein Atlas. *Nature biotechnology* 28, 1248 (Dec, 2010).
16. B. Davidson *et al.*, Gene expression signatures differentiate ovarian/peritoneal serous carcinoma from breast carcinoma in effusions. *Journal of cellular and molecular medicine* 15, 535 (Mar, 2011).
17. L. E. Kelemen, The role of folate receptor alpha in cancer development, progression and treatment: cause, consequence or innocent bystander? *International journal of cancer. Journal international du cancer* 119, 243 (Jul 15, 2006).
18. K. R. Kalli *et al.*, Folate receptor alpha as a tumor target in epithelial ovarian cancer. *Gynecologic oncology* 108, 619 (Mar, 2008).

19. S. Markert *et al.*, Alpha-folate receptor expression in epithelial ovarian carcinoma and non-neoplastic ovarian tissue. *Anticancer research* 28, 3567 (Nov-Dec, 2008).

APPENDICES:

Available Upon Request: Supplementary Table S1. List of ovarian cancer cell surface N-glycoproteins

TABLES & FIGURES:
Listed in Order Below

Table 1. Total and cell surface proteins identified from cell surface capture (CSC) of OVCAR3 and Serous Ovarian Cancer (SOC) cells

		Number of proteins	Membrane proteins by GO	Membrane proteins by TMHMM	Surface proteins
OVCAR3	With N~XS/T	275	172	228	235
	No N~XS/T	196	24	50	14
Total		471	196	278	249
SOC	With N~XS/T	402	239	325	300
	No N~XS/T	73	12	10	3
Total		475	251	335	303
Unique Proteins (OVCAR3 + SOC combined)	With N~XS/T	519	305	415	394
	No N~XS/T	253	34	58	17
Total		772	339	473	411

Table 2. Candidate targets for ovarian cancer molecular imaging probes.

Gene symbol	FOLR1	MFGE8	ITGB6	TACSTD2	CSPG5	ST14
Contains N~XS/T motif	Y	Y	Y	Y	Y	Y
Cell surface localization by GO	Y	N	Y	Y	Y	Y
Membrane Helix by TMHMM	Y	N	Y	Y	Y	Y
Relative expression in SOC tissue vs. normal ovary (mRNA)	3.07	0.46	1.89	2.83	2.05	2.6
Relative expression in SOC tissue vs. normal fallopian tube (mRNA)	0.98	1.07	2.23	1.36	0.47	0.99
Detected in plasma from healthy women by LC-MS/MS	N	Y	N	N	N	N
Normal tissues with "high expression"* (mRNA)	Salivary gland, Lung, Trachea	Heart, Ovary, Prostate, Trachea, Bladder, Uterus	Pancreas, Ovary Breast, Kidney, Bone marrow	Skin, Trachea, Breast, Salivary gland	Brain, Spinal Cord	Colon, Intestine, Salivary gland, Trachea, Uterus
Number of normal tissue types demonstrating protein expression as reported by The Human Protein Atlas	Not reported	13 out of 64	35 out of 66	Not reported	Not reported	Not reported

*defined as tissues with expression levels exceed 1 SD above the mean level for all genes in all tissues.

Table 3. Expression of candidate targets by immunohistochemistry in benign ovarian tumors, normal ovary and fallopian tube relative to high grade SOC.

	High Grade SOC	Benign Tumor	Normal Ovary (premenopausal)	Normal Ovary (postmenopausal)	Normal FT (premenopausal)	Normal FT (postmenopausal)
FOLR1	++	-	-	-	-	-
MFGE8	++	++	+	-	++	++
ITGB6	+++	++	++	++	+++	+++
TACSTD2	++	++	-	-	++	++
CSPG5	+	+	+	+	+	+
ST14	++	++	+	+	++	++

Figure 1. Molecular function categories of surface proteins identified by cell surface capture (CSC) from (a) OVCAR3 and (b) SOC cells compared to (c) proteins identified by LC-MS/MS shotgun analysis of OVCAR3 whole cell lysates.

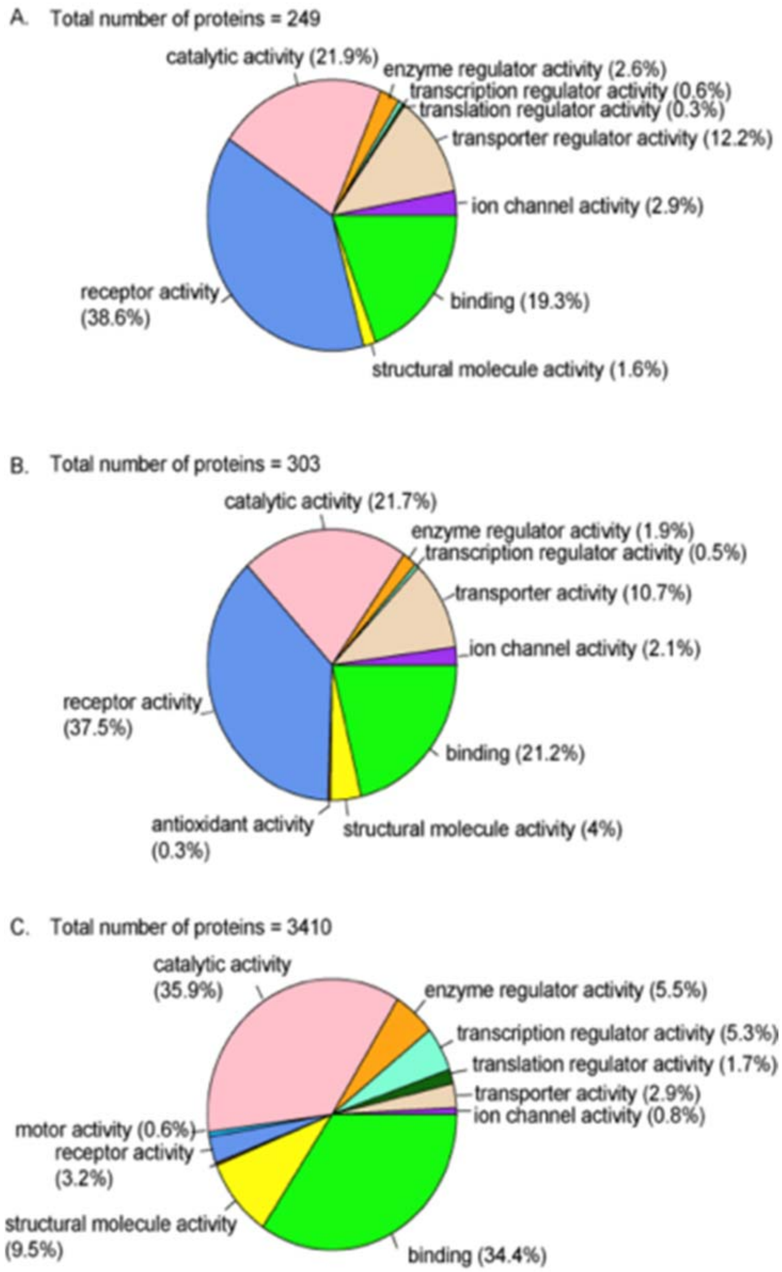

Figure 2. Expression of candidate markers on the cell surface of serous ovarian cancer tissue by immunohistochemistry. Representative image from one of 10 tumors studied.

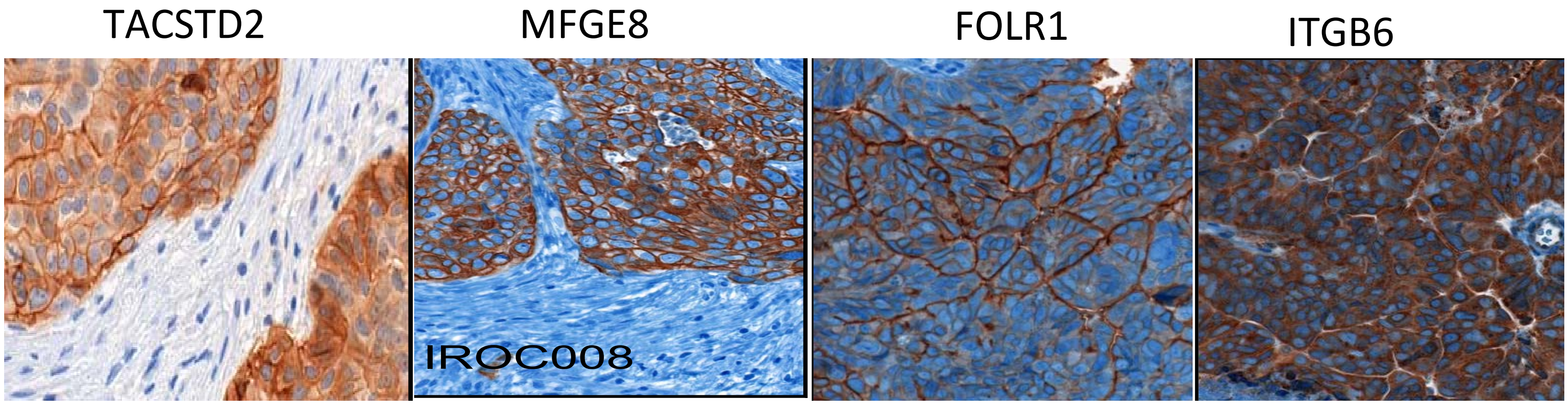

Figure 3. FOLR1 expression in high grade invasive serous cancer, serous borderline tumor and benign and normal ovary and fallopian tube. Representative images from 1 of 5 tissues stained. a) High grade invasive serous cancer, b) Serous borderline tumor, c) benign dermoid, d) normal ovary, e) normal pre-menopausal FT, and d) normal post-menopausal FT. FOLR1 stains only the cancer.